Donor age intensifies the early immune response after transplantation

A Reutzel-Selke[1], A Jurisch[1], C Denecke[1], A Pascher[1], PNA Martins[1], H Keßler[2], A Tamura[3], N Utku[3], J Pratschke[2], P Neuhaus[2] and SG Tullius[1,3]

[1]Department of General, Visceral, and Transplantation Surgery, Charité-Campus Virchow Clinic, Universitätsmedizin Berlin, Berlin, Germany; [2]Institute of Medical Immunology, Charité-Campus Mitte, Universitätsmedizin Berlin, Berlin, Germany and [3]Division of Transplant Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA

Increasing donor age is associated with reduced graft function. We wondered if donor age may not only affect intrinsic function but also alter the immune response of the recipient. Kidneys from young and old F-344 rats (3 vs 18 months) were transplanted into bilaterally nephrectomized young Lewis recipients and compared with age-matched controls (follow-up: 6 months). Renal function and structural changes were assessed serially in both native kidneys and allografts. Host alloreactivity, graft-infiltrating cells, and their inflammatory products were determined at intervals to examine the correlation of immune response and donor age. Functional and structural deterioration had advanced significantly in older allografts compared with age-matched native controls, whereas differences between young allografts and native controls of similar age were only minor. Changes in grafts from elderly rats were associated with a more intense host immune response early post-transplant (up to 1 month) reflected by significantly higher numbers of peripheral T and B cells, increased T-cell alloreactivity and modified cytokine patterns associated with elevated frequencies of intragraft dendritic cells, B cells, and CD31+ cells. By 6 months, recipients of young donor grafts produced comparable or more intense alloantigen-specific immune responses. Older donor grafts elicit a stronger immune response in the early period after transplantation.

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Correspondence: *SG Tullius, Division of Transplant Surgery, Brigham and Women's Hospital, Harvard Medical School, 75 Francis St, Boston, Massachusetts 02115, USA. E-mail: stullius@partners.org*

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The growing organ shortage represents the largest problem in organ transplantation. As a consequence, increased numbers of so-called 'marginal' or extended criteria donor grafts are utilized. Although a common and broadly accepted definition for the term extended criteria donor has not been coined, various donor criteria, including comorbid disease, intensive care treatment, brain death, organ harvesting, and prolonged ischemia have been identified as relevant risk factors. Additionally, increased donor and recipient age have been shown as independent risk factors for the development of chronic graft dysfunction in clinical and experimental studies.[1]

Studies in the European-Senior-Program (ESP) suggested that age matching of donor and recipient may have a beneficial effect on graft outcome. The engraftment of kidneys from older donors into elderly recipients with an ameliorated immune response may allow physiological matching.[2–4] However, our initial clinical experience with grafts from elderly donor grafts and recipients with less potent, calcineurin-inhibitor (CNI)-free immunosuppression demonstrated increased rates of acute rejection episodes requiring frequent conversion to more potent immunosuppressants.[5]

Our own previous experimental data revealed an acceleration of chronic graft dysfunction with increasing donor and recipient age.[6] These results may be dependent on a modified immune response in older recipients, particularly during the early post-transplant period. On the other hand, grafts from older donors may elicit an accelerated immune response due to elevated immunogenicity and reduced resistance to unspecific damages.

An increased incidence of acute rejection episodes associated with transplantation of older donor grafts may influence long-term function.[7–9] In addition, a reduced capability for repair and renewal of damaged tissue may result in a persistent inflammation in parallel to increased antigen recognition.[10–13] Moreover, several clinical and experimental studies demonstrated an increased allostimulation by 'aged' antigen-presenting cells (APC) and enhanced immune responses following the contact with aged APC.[14,15]

In the current study, we dissected the effects of donor age on graft immunogenicity and consecutive alterations of the

recipient's immune response in an established experimental kidney transplantation model.

RESULTS

Recipients of grafts from both young and old donors survived the observation period. To correlate age-related physiological changes with alterations in age-matched allografts, we measured the increment of functional/structural deterioration.

Renal function

Proteinuria in old native uni-nephrectomized controls increased twofold over the 6 months observation period, while rising almost fivefold during this time period in older allografts (increase: $P = 0.0003$; old vs young allograft by month 6: $P = 0.0015$; Figure 1, Table 1). In contrast, proteinuria remained stable in both, young native controls and recipients of young donor grafts ($P =$ NS).

Creatinine clearance (CL) was significantly lower in recipients of older donor grafts at the end of the observation period ($P = 0.0093$), whereas no significant differences were observed between young and old nephrectomized controls (Table 1).

Morphology

Morphological criteria were not significantly different between 3- and 18-month-old native controls. However, the ratio of structural changes had significantly progressed in older donor grafts/older age-matched native controls vs younger donor grafts/younger age-matched controls (Figure 2a–d, Table 1).

Alloantigen-specific intracellular IFN-γ secretion enzyme-linked immunosorbent assay

The immune response of recipients of elderly donor grafts was more intense than that of recipients of younger donor grafts. Although levels of intracellular interferon (IFN)-γ increased during the observation period in both recipients of young and old donors, grafts from older donors elicited higher frequencies of alloreactive T cells ($P = 0.0022$ by month 1, and $P = 0.04$ by month 3, respectively; Figure 3).

Cytokine production after *in vitro* stimulation of splenocytes ELISA

Production of interleukin (IL)-2, IFN-γ, IL-4, and IL-10 was significantly elevated in recipients of old donor grafts early

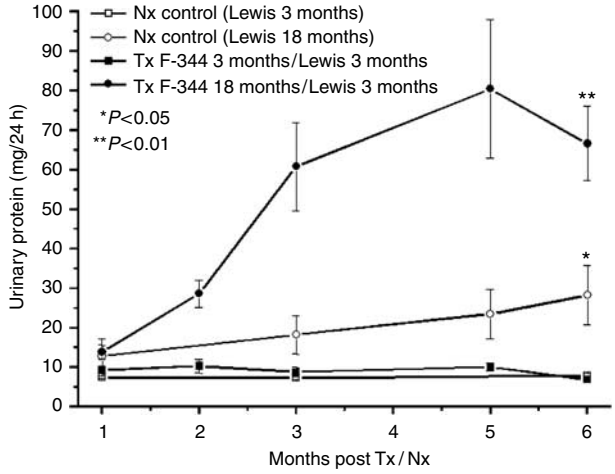

Figure 1 | Urinary protein secretion (mg/24 h) in young LEW recipients following the transplantation (Tx) of young or old F-344 donor kidneys. Age-matched nephrectomized (Nx) animals served as controls. While proteinuria in old vs young native age-matched nephrectomized controls increased twofold from month 1 to 6, proteinuria in recipients of old donor kidneys increased almost fivefold during this time period compared with recipients of young donor organs.

Table 1 | Renal function (proteinuria, creatinine clearance) and structural changes (glomerulosclerosis, arteriosclerosis, tubular atrophy, fibrosis, cellular infiltration) following the transplantation (Tx) of young or old F-344 donor kidneys into young Lewis recipients compared to age-matched nephrectomized (Nx) control animals

Renal function and structural changes 6 months post-Nx/Tx	Nx: control age			Tx: donor age		
	3 months	vs	18 months	3 months	vs	18 months
Urinary protein (mg/24 h) *1 month post-Nx/Tx*	7.5±0.7.	NS	12.8±4.4 *(NS vs 6 months)*	9.3±0.8	*P=0.0421*	13.9±1.2 *(P=0.0003 vs 6 months)*
Urinary protein (mg/24 h) *6 month post-Nx/Tx*	7.9±0.5	*P=0.0159*	26.3±4.1	7.0±0.9	*P=0.0015*	66.6±9.4 *(P=0.0107 vs Nx control)*
Creatinine clearance (ml/min)	1.96±0.20	NS	1.72±0.42	1.98±0.19	*P=0.0093*	1.25±0.14
Glomerulosclerosis (%)	20±3	NS	36±14	26±4	*P=0.0317*	52±8
Arteriosclerosis[a]	1.9±0.1	NS	2.0±0.1	2.1±0.1	NS	2.3±0.2
Tubulary atrophy[a]	1.7±0.2	NS	1.5±0.5	1.4±0.2	*P=0.0061*	2.5±0.2 *(P=0.0195 vs Nx control)*
Fibrosis[a]	1.0±0.3.	NS	0.7±0.3	0.3±0.2	*P=0.0075*	1.9±0.4 *(P=0.072 vs Nx control)*
Cellular infiltration[a]	1.0±0.2	NS	1.5±0.3	0.6±0.2	*P=0.0004*	2.5±0.2 *(P=0.0429 vs Nx control)*

NS, nonsignificant; Nx, nephrectomy; Tx, transplantation.
[a]The extent of arteriosclerosis, tubular atrophy, interstitial fibrosis, and cellular infiltrates was quantified on a 0 to 4+ scale (4+=greatest structural deterioration / >20 fields of view (fv) per section were evaluated at ×200).

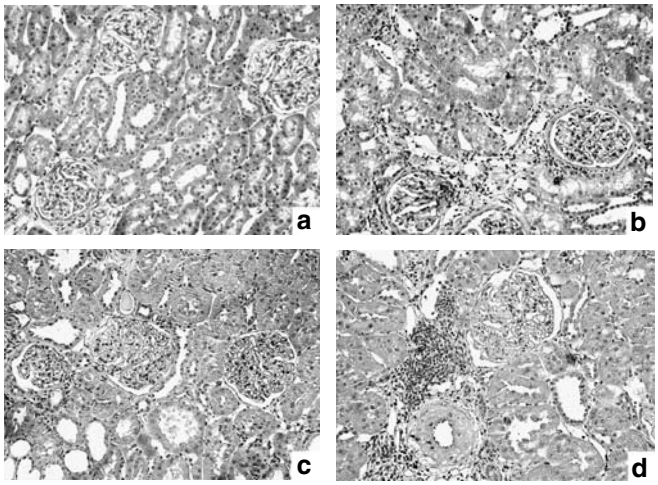

Figure 2 | Structural changes 6 months post-nephrectomy/transplantation (Nx/Tx). (**a**) Nx control/3 months old at time of Nx, (**b**) Nx control/18 months old at time of Nx, (**c**) Tx/donor age 3 months, and (**d**) Tx/donor age 18 months. Structural changes had significantly progressed in older grafts after 6 months (representative graft sections following hematoxylin and eosin staining, original magnification $\times 200$).

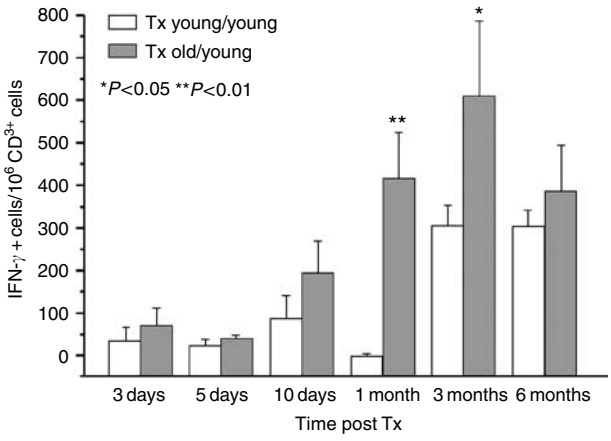

Figure 3 | T cell alloreactivity. Secretion of intracellular IFN-γ following donor-specific *in vitro* stimulation of recipient splenocytes (ELISPOT). Although levels of intracellular IFN-γ increased during the observation period in both, recipients of young and old donors, older grafts elicited higher frequencies of alloreactive T cells at all time intervals.

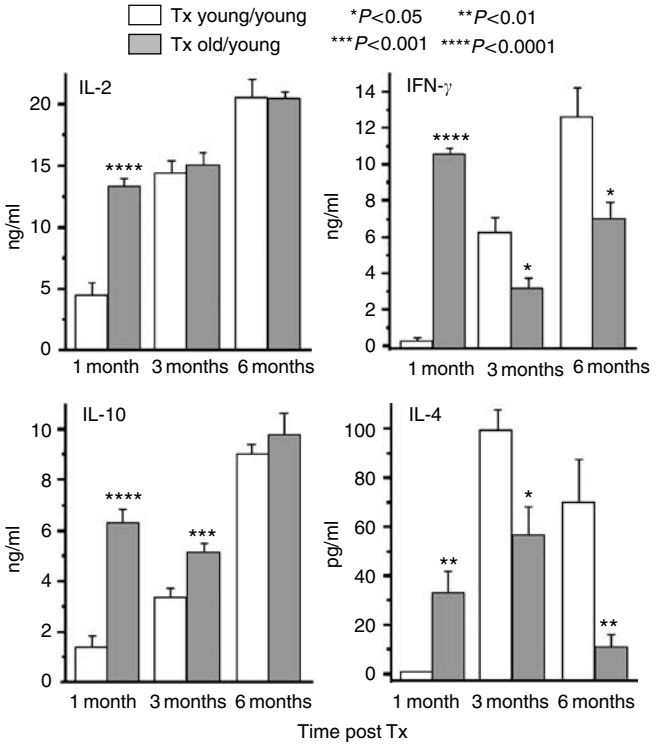

Figure 4 | Serial analysis of cytokine production following *in vitro* stimulation of recipient splenocytes (enzyme-linked immunosorbent assay). Production of IL-2, IFN-γ, IL-4, and IL-10 increased significantly in recipients of old grafts 1 month post-Tx. In contrast, after 6 months levels of IFN-γ and IL-4 had increased in recipients of grafts from young donors, whereas the production of IL-2 and IL-10 was comparable in both study groups at the end of the observation period.

after transplantation (by 1 month: *IL-2*: $P<0.0001$;. *IFN-γ*: $P<0.0001$; *IL-4*: $P=0.0027$; *IL-10*: $P<0.0001$). However, at the end of the observation period levels for IFN-γ and IL-4 had increased in recipients of young donor grafts (*IFN-γ*: $P=0.0128$; *IL-4*: $P=0.0045$; Figure 4).

T-cell subsets, B cells, and DC in peripheral organs

Recipients of older donor grafts displayed significantly increased amounts of CD3 + CD4 + T cells in spleens early after transplantation ($P=0.028$ and 0.017 by days 5 and 10, respectively). In parallel, CD3 + CD8 + cells had increased in peripheral blood and spleens in recipients of elderly donor grafts ($P=0.0012$ and 0.0535, respectively, by day 3; data not shown). Peripheral blood immunoglobulin (Ig)κ + B cells had significantly increased in recipients of older donor grafts at early time intervals ($P=0.0001$ by day 5, and $P=0.0049$ by 1 month, respectively; Figure 5). By 6 months, however, frequencies of T and B cells were comparable in all compartments. The presence of dendritic cells (DC) in peripheral compartments (spleen, lymph nodes, blood) was not influenced by donor age at any time.

Graft-infiltrating cells

Intragraft OX62 + DC were elevated in recipients of elderly grafts at early time intervals ($P=0.011$ and 0.016 by day 3 and 1 month, respectively). At later time intervals (3 and 6 months) frequencies of intragraft DC were not different in young and old donor transplants (Figure 6).

The majority of graft-infiltrating cells expressing high levels of major histocompatibility complex (MHC) class II and the costimulatory molecule CD86 were found within the Igκ + B-cell population. These cells were significantly

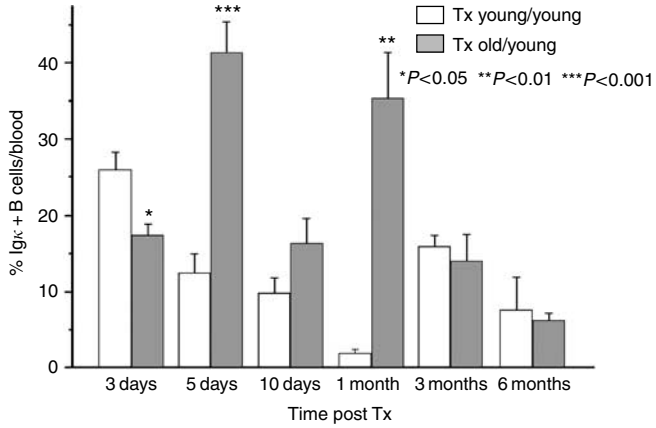

Figure 5 | Fluorescence-activated cell sorter analysis of recipient peripheral blood mononuclear cells. Cells were stained with fluorescein isothiocyanate-conjugated mAb OX-12 specific for rat Igκ + B cells. Recipients of older grafts demonstrated significantly higher frequencies of peripheral blood B cells at early time intervals.

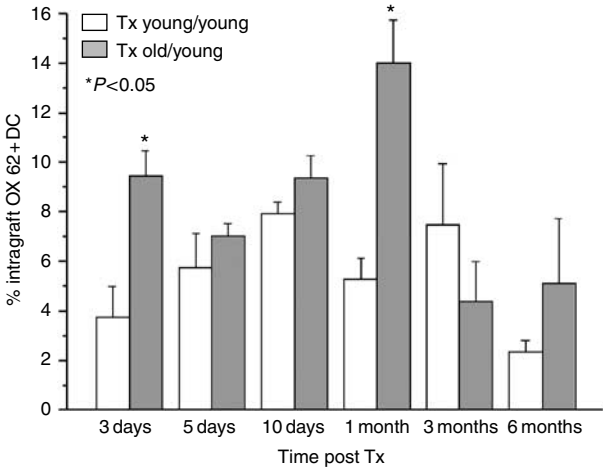

Figure 6 | Serial fluorescence-activated cell sorter analysis of intragraft DC. Cells were stained with fluorescein isothiocyanate-conjugated mAb OX-62 specific for rat DC. Intragraft OX62 + DC were significantly elevated in recipients of older grafts early post-transplantation.

increased in grafts from older donors (*Igκ + B cells*, $P = 0.001$ and 0.065; *Igκ + MHC class II + cells*, $P = 0.035$ and 0.063; *Igκ + CD86 + cells*, $P = 0.009$ and 0.022, by days 3 and 5, respectively; Figure 7a–c). Intragraft B cells peaked by day 10 in both, recipients of young and old donor grafts, albeit not showing quantitative significant differences at that time, and declined significantly at later time intervals ($P < 0.05$). The endothelial marker platelet-endothelial cell adhesion molecule (PECAM)-1 (CD31) was significantly higher expressed in older donor grafts early after transplantation (*CD31 + cells*, $P = 0.0016$, and *CD31 + MHC class II + cells*, $P = 0.0008$ by day 3, not shown).

Thus, overall graft-infiltrating cells and activation markers were more pronounced early after transplantation in kidneys from older donors, whereas stronger in younger donor kidneys at later time intervals.

DISCUSSION

Donor age is an important predictor of long-term graft outcome.[10,16,17] Grafts from older donors demonstrate an increased incidence of acute rejection episodes in the early post-transplantation period[7–9,18] leading to subsequent chronic graft deterioration. Pre-existing donor criteria such as age, previous diseases, and brain death as well as perioperative injuries may influence long-term graft survival in an antigen-independent, nonspecific manner.[19]

The aging process causes important changes that result in a diminution of the functional reserve and inability to respond adequately to stress conditions.[10,20] Recently, Halloran *et al.*[10] introduced the term 'senescence', a normal process in the cell cycle to sort out exhausted cells and prevent malignant mutations. Recent studies on cell biology, aging, and senescence described the limitation of cell cycles of somatic cells and telomere shortening with age in humans. These processes, which finally lead to the shut down of the cell, may play an important role in cellular senescence and may explain the incompetence of aged organs to repair damaged tissue.[10,11] Indeed, grafts from older donors show an impaired ability to repair injured tissue, which in turn

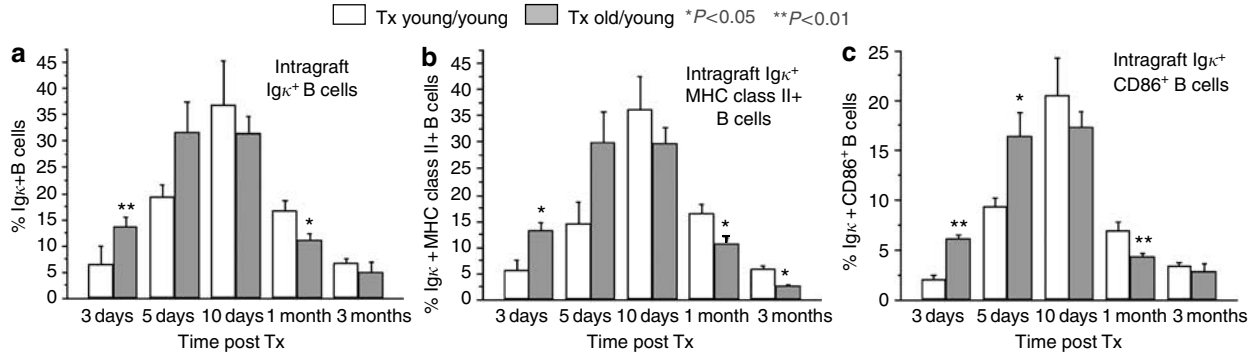

Figure 7 | Serial fluorescence-activated cell sorter analysis of graft-infiltrating cells. (**a–c**) Cells were stained with conjugated mAbs specific for Igκ + B cells, CD86, and MHC class II. Old grafts demonstrated significantly increased frequencies of intragraft B cells expressing high levels of MHC class II and costimulatory molecule CD86 at early time intervals.

may lead to persisting inflammation and increased immune recognition. Previously, Melk *et al.*[21] described mechanisms of cell senescence in aged rat kidneys independent of telomere shortening, which may contribute to nephron shutdown and an age-related pathology of rat kidneys. In keeping with the observation that immunosenescence and many conditions leading to end stage renal disease share common features of functional and structural decline, age-related progressive fibrosis of both interstitium and glomeruli may lead to enhanced infiltration of immune cells, thereby promoting a proinflammatory environment.[10,22] Besides, primarily cortical loss of functional nephrons and increased vascular resistance might further contribute to hyperfiltration and proteinuria, which in turn lead to tubular cell activation, gene expression of inflammatory mediators, and ongoing immune response in aged kidneys. Accordingly, 18-month-old rats displayed an almost normal GFR followed by a continuously regressive filtration rate and total loss of functional reserve beyond this age.[23]

Thus, it can be hypothesized that immunosenescence and functional alterations with advancing age act synergistically in stimulating unspecific inflammation, progressive functional loss, and immune recognition. In regard to the 'injury response', antigens expressed on injured grafts are more likely to activate and provoke an immune response.[9] This process is associated with increased expression of proinflammatory cytokines, MHC antigens, and increased recruitment and activation of APC.[12,13] Several clinical and experimental studies report on an increased allostimulation by aged APC and enhanced immune responses following the contact with 'aged' APC.[14,15] DC are the most potent stimulators of T cells and are responsible for initiating an immune response by alloantigen presentation to naive T cells. Several authors previously reported an impaired production of IL-1 by peritoneal APC,[24] altered levels of macrophage products,[25] and dysregulation of macrophage-derived IL-12, IL-10, and IFN-γ production with increasing age.[26,27] In addition, an age-related impaired T-cell proliferation associated with enhanced APC function and increased production of IL-10 and IL-12 has been reported.[15,28]

Chronic allograft deterioration is a multi-faceted process. The F-344 – Lewis (LEW) kidney transplant model has been used before by us and others to study the events of this process.[29,30] This model has been characterized with differences in two MHC class I loci and various non-MHC genes. An initial immunosuppression is necessary in this model to suppress early acute rejection episodes. However, long-term immunosuppression is not applied, thus differing from the clinical situation.

Our experimental data demonstrate the induction of a strong immune response in the early post-transplantation period after engraftment of aged donor kidneys leading to long-term functional and structural deterioration. While a restricted physiological capacity of older grafts has been assumed, we were able to show a modified immune response with increasing donor age.

Although early functional measurements were not obtained in our current experiment, donor age did not impact early renal function (by 2 weeks) in a previous study.[19] Thus, senescence rather than consequences of unspecific injuries associated with ischemia or delayed graft function (DGF)[31,32] seems to be causative for the modified immune response observed in our model.

Alloreactive T cells increased during the observation period in both recipients of young and old donor grafts. However, T-cell alloreactivity was significantly elevated in young recipients of old grafts after allospecific *in vitro* stimulation with splenocytes of donor origin. Recipients of older donor grafts demonstrated increased levels of IL-2, IFN-γ, IL-4, and IL-10 early after transplantation. At the end of the observation period, levels were comparable (IL-2, IL-10) or showed a significant increase (IFN-γ, IL-4) in recipients of young grafts at this time. T cell (CD3, CD4, CD8) and Igκ+ B cell counts were significantly elevated in peripheral compartments early post-transplantation when kidneys from older donors were engrafted accompanied by higher frequencies of intragraft OX62+ DC. Again, those differences declined by the end of the observation period.

Furthermore, we observed a strong intragraft B-cell accumulation in older grafts early after transplantation with a strong expression of MHC class II, the costimulatory molecule CD86, and high frequencies of intragraft CD31+ MHC class II+ cells.

These effects may be related to an age-associated increase in graft immunogenicity.

Thus, further investigations have to focus on the mode of antigen presentation and the role of 'passenger leukocytes' within the 'old' graft potentially provoking a subsequent stronger T-cell immune response in the young recipient.

Recognition of foreign antigens can occur by two distinct, but not exclusive pathways. The direct pathway represents the recognition of foreign MHC molecules on the surface of donor APC. The indirect pathway occurs when donor MHC molecules are internalized, processed, and presented to alloreactive T cells by host APC.[33] There is evidence that donor DC ('passenger leukocytes') promote graft acceptance by the indirect pathway. On the other hand, donor DC were suggested to increase graft immunogenicity and to trigger acute rejection by the direct pathway.[34] The primary presentation of donor antigen by the direct pathway in combination with 'aged' donor-derived APC may lead to an enhanced immune response, particular during the early period after transplantation. It can be speculated that donor-derived APC will be depleted from the transplanted organ within weeks after transplantation, whereas recipient APC permanently migrate through the graft.

In fact, our own results demonstrated an increase in T-cell alloreactivity, cytokine production and higher numbers of peripheral T and B cells early after transplantation in recipients of older grafts, whereas differences in the immune response to young vs old grafts were diminished at later time points.

An interesting finding in our study was the presence of an increased B-cell infiltration in grafts from older donors. Recent clinical studies described dense clusters of B cells in kidney graft biopsies strongly associated with both severity and steroid resistance of rejection episodes, suggesting an important role of infiltrating B cells in acute rejection and graft outcome.[35,36] Moreover, a recent case report presented data on acute rejection episodes with increased frequencies of CD20+ B cell infiltrates refractory to standard immunosuppressive therapy. Consequently, graft function had improved after treatment with rituximab, a humanized anti-CD20 monoclonal antibody (mAb).[37,38] B-cell infiltration was not correlated with humoral-mediated graft injury as elevated levels of Ig or complement deposits were absent.[35] Recent studies by Steinmetz *et al.*[39] propose that CXCL13-mediated attraction of CXCR5-expressing B cells into rejecting allografts play a crucial role in B-cell cluster formation and predict chronic allograft rejection. However, the functional relevance of intragraft B cells needs further investigation. Besides the production of alloantibodies, B cells may play a pivotal role as APC in indirect antigen recognition.

An additional finding in our studies was the high intragraft frequency of CD31+ MHC class II+ cells shortly after transplantation of aged grafts in young recipients. The CD31 mAb stains for the PECAM-1, which is expressed by endothelial cells, platelets, monocytes, neutrophils, and certain T-cell subsets and is involved in acute and chronic allograft rejection via its effects on leukocyte trafficking.[40–42] MHC class II molecules are upregulated on endothelial cells in allografts. Interaction between alloreactive CD4+ T cells and activated endothelial cells expressing MHC class II may play a role in recruitment of leukocytes into vascularized allografts and may provide antigen-dependent signals through the direct pathway.[42–44] Indeed, increased intragraft PECAM-1 (CD31) and MHC class II expression in older grafts was associated with enhanced leukocyte infiltration (T cells, B cells, and DC) in our experiments. Although previous studies showed an increased expression of PECAM-1 on monocytes and leukocyte function-associated molecule-1 (CD11a) on lymphocytes with increasing age,[45] others report on age-related declines in numbers of blood mononuclear and vascular endothelial cells, structural and functional alterations, and a senescence-associated decline of lymphocyte migration.[46–48]

In conclusion, our results suggest that grafts from older donors are more immunogenic and therefore recipients of aged kidneys may require a more intense immunosuppression in the early period after transplantation. Donor treatment may be particularly beneficial in older grafts.

MATERIALS AND METHODS

The transplantation model

Male inbred rats (Harlan Winkelmann, Borchen, Germany) were used throughout the experiments. Kidneys from young and old Fischer 344 rats (F-344, RT1^{1v1}, 3-month-old, 235±30 g, and 18-month-old, 430±30 g, respectively) were transplanted into LEW recipients (RT1^{1}, 3-month-old, 275±40 g) using standard microsurgical techniques (anastomosis time 25±5 min). One native kidney was removed during transplantation, and the remaining kidney 10 days later; cyclosporine A treatment was given for 10 days at a dosage of 1.5 mg/kg/day to prevent initial acute rejection episodes. Before organ harvesting, grafts were perfused with University of Wisconsin solution and kept at 4°C for 5 min. Graft survival and renal function (urinary protein excretion, creatinine clearance) were monitored for 6 months. Data were compared with native, uni-nephrectomized young and old controls to distinguish age-related physiological changes at the beginning (3 and 18 months) and at the end of the observation period (9 and 24 months), respectively. Donor age-related immune response was analyzed sequentially in graft sections, blood samples, and spleen of allograft recipients taken by 3, 5, 10 days and 1, 3, and 6 months ($n = 6$/group).

All animal experiments were performed with the permission of the local authorities (Landesamt für Gesundheitsschutz, Arbeitsschutz und Technische Sicherheit, Berlin, Germany, Project-No.: G 0096/03).

Renal function

Serum and urine samples were collected in all groups. Creatinine clearance was tested at monthly intervals and calculated as: urine creatinine (mg/ml) × urine volume (ml)/serum creatinine (mg/ml) × time of urine collection (min).

Protein excretion (mg/24 h) was measured by precipitation with 20% CCl_3COOH. Turbidity was assessed at a wavelength of 415 nm using a Hitachi 911 analyzer.

Proteinuria, the most sensitive parameter for chronic renal allograft nephropathy in rat models,[49] was tested at similar intervals. The relative increase in urinary protein excretion between 1 and 6 months was calculated in recipients of grafts from older vs younger donors and compared with the increase in protein excretion in age-matched unilaterally nephrectomized controls.

Morphological analysis

Structural changes in allografts were compared with native kidneys in age-matched uni-nephrectomized controls. Specimens were fixed in 4% buffered formalin, paraffin embedded, stained with hematoxylin and eosin, and assessed by light microscopy. To determine the extent of glomerulosclerosis, the number of glomeruli per kidney section were counted and the ratio was expressed as a percentage of affected to normal glomeruli. The extent of arteriosclerosis, cellular infiltrates, tubular atrophy, and interstitial fibrosis was quantified on a 0 to 4+ scale (4+ = greatest structural deterioration / >20 fields of view per section were evaluated at ×200).

Flow cytometry

Heparinized blood and biopsies of spleen and graft sections were obtained from LEW recipients at sequential time intervals. Graft sections were pretreated with collagenase IV (1%/RPMI) for 30 min. Peripheral blood mononuclear cells, splenocytes, and graft-infiltrating cells were isolated by a standard procedure using Pancoll™ density gradient centrifugation (density 1.091 g/ml; Pan Biotech GmbH, Aidenbach, Germany). After isolation, cells were resuspended in RPMI 1640 containing 10% fetal calf serum, 1% glutamine, and 1% penicillin/streptomycin at a concentration of 5×10^6 cells/ml. Aliquots (400 µl) of the cell suspension were distributed for staining.

Mouse anti-rat mAbs (BD PharMingen GmbH, Heidelberg, Germany; Biocarta Europe GmbH, Hamburg, Germany) used included CD3-PE (IF4, T cells), CD4-APC (W3/25, T-helper cells), CD8-PerCP (OX-8, cytotoxic T cells), Igκ-fluorescein isothiocyanate (OX-12, Pan-B cells), OX-62-PE (OX-62, rat DC), CD86-PE (24F, B7-2, activated B cells, and monocytes), MHC class II-PerCP (OX-6, RT1B, activated APC), and CD31-PE (TLD-3A12, platelet endothelial cellular adhesion molecule/PECAM-1, leukocytes, and endothelial cells).

Fixed and stained lymphocyte samples were analyzed with a FACScalibur, (Becton Dickinson, Palo Alto, CA) and analyzed with CELLQuest software. The cytometer was calibrated with CaliBRITE1 beads (Becton Dickinson, USA) using FACSComp software and with QC-3 beads according to the manufacturer's recommendations (Flow Cytometry Standards Corp., San Juan, Puerto Rico). A total of 500 000 events were acquired.

In vitro stimulation of rat splenocytes

Single cell suspensions of splenocytes were prepared in RPMI medium with 10% fetal calf serum and dispensed into 24-well plates at a concentration of 1×10^7/well. Cells were stimulated with Lipopolysaccharide (LPS: 10 μg/ml), Concavalin A (ConA: 5 μg/ml) for 5 h or phorbol 12-myristate 13-acetate (PMA 2 ng/ml)/ionomycin (1 μg/ml) for 24 h at 37°C. Unstimulated rat spleen cells served as controls. Supernatants of spleen cell cultures were tested for IL-2, IFN-γ, IL-4, and IL-10 using enzyme-linked immunosorbent assay kits as recommended by the manufacturer (OptEIA™ Set, Pharmingen, San Diego, CA, USA).

Alloreactive ELISPOT

The frequency of alloreactive T cells was determined in LEW recipients using a rat IFN-γ ELISPOT assay (Diaclone, Besançon Cedex, France). Rat spleen cells were prepared as described above. Briefly, polyvinylidine difluoride (PVDF)-bottomed 96-well microtiter plates (Diaclone) were incubated with 70% ethanol for 10 min. Plates were incubated with an anti-rat IFN-γ capture antibody for 12 h; unspecific binding was blocked with 2% skimmed dry milk in phosphate-buffered saline. Subsequently, RPMI medium (negative control), PMA (2 ng/ml/2 μg/ml, both Sigma Aldrich GmbH, Munich, Germany), or 5×10^5 stimulator cells (F-344 splenocytes) were dispensed into the wells. Thereafter, 5×10^5 responder cells (LEW splenocytes) were added. Plates were incubated for 24 h, washed and incubated with a biotinylated anti-rat IFN-γ detection antibody for 3 h. After a final 45 min incubation period with a streptavidin-alkaline phosphatase conjugate and subsequent administration of the staining buffer (BCTP/NTB; Diaclone), plates were dried and alloreactive T cells were measured using the BIOREADER® 3000 C/BIOCOUNTER system (BIOREADER 3000 C, BIOSYS GmbH, Karben, Germany). The actual frequency was determined as a ratio of the number of spots/number of responder cells. This ratio was then adapted to the actual percentage of T cells in the responder cell preparation assessed by flow cytometry.[50]

Statistics

Data were expressed as mean $\pm$ s.e. for comparison using unpaired *t*-test or Mann–Whitney Test, respectively. Statistical difference was accepted at $P < 0.05$.

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REFERENCES

1. Martins PN, Pratschke J, Pascher A *et al.* Age and immune response in organ transplantation. *Transplantation* 2005; **79**: 127–132.
2. Theodorakis J, Schneeberger H, Illner WD *et al.* Nephrotoxicity free, mycophenolate mofetil-based induction/maintenance immunosuppression in elderly recipients of renal allografts from elderly cadaveric donors. *Transplant Proc* 2000; **32**(Suppl 1A): S9–S11.
3. Jassal SV, Opelz G, Cole E. Transplantation in the elderly: a review. *Geriatr Nephrol Urol* 1997; **7**: 157–165.
4. Bradley BA. Rejection and recipient age. *Transplant Immunol* 2002; **10**: 125–132.
5. Fritsche L, Horstrup J, Budde K *et al.* Old-for-old kidney allocation allows successful expansion of the donor and recipient pool. *Am J Transplant* 2003; **3**: 1434–1439.
6. Pascher A, Reutzel-Selke A, Jurisch A *et al.* Alterations of the immune response with increasing recipient age are associated with reduced long-term organ graft function of rat kidney allografts. *Transplantation* 2003; **76**: 1560–1568.
7. Cho YW. Expanded criteria donors. *Clin Transplant* 1998: 421–436.
8. Kerr SR, Gillingham KJ, Johnson EM *et al.* Living donors >55 years: to use or not to use? *Transplantation* 1999; **67**: 999–1004.
9. de Fijter JW, Mallat MJ, Doxiadis II *et al.* Increased immunogenicity and cause of graft loss of older donor kidneys. *J Am Soc Nephrol* 2001; **12**: 1538–1546.
10. Halloran PF, Melk A, Barth C. Rethinking chronic allograft nephropathy: the concept of accelerated senescence. *J Am Soc Nephrol* 1999; **10**: 167–181.
11. Melk A, Halloran PF. Cell senescence and its implications for nephrology. *J Am Soc Nephrol* 2001; **12**: 385–393.
12. Shoskes DA, Parfrey NA, Halloran PF. Increased major histocompatibility complex antigen expression in unilateral ischemic acute tubular necrosis in the mouse. *Transplantation* 1990; **49**: 201–207.
13. Penfield JG, Dawidson IA, Ar'Rajab A *et al.* Syngeneic renal transplantation increases the number of renal dendritic cells in the rat. *Transplant Immunol* 1999; **7**: 197–200.
14. Ordemann R, Hutchinson R, Friedman J *et al.* Enhanced allostimualtory activity of host antigen-presenting cells in old mice intensifies acute graft-versus-host disease. *J Clin Invest* 2002; **109**: 1249–1256.
15. Castle SC, Uyemura K, Crawford W *et al.* Antigen presenting cell function is enhanced in healthy elderly. *Mech Ageing Dev* 1999; **107**: 137–145.
16. Terasaki PI, Cecka JM, Gjertson DW. Impact analysis: a method for evaluating the impact of factors in clinical renal transplantation. *Clin Transplant* 1998: 437–441.
17. Gjertson DW. A multi-factor analysis of kidney regraft outcomes. *Clin Transplant* 2002: 335–349.
18. De Fijter JW, Persijn GG. Age, the riddle of renal transplantation. *Nephrol Dial Transplant* 2005; **20**: 2307–2310.
19. Tullius SG, Reutzel-Selke A, Egermann F *et al.* Contribution of prolonged ischemia and donor age to chronic renal allograft dysfunction. *J Am Soc Nephrol* 2000; **11**: 1317–1324.
20. Epstein M. Aging and the kidney. *J Am Soc Nephrol* 1996; **7**: 1106–1122.
21. Melk A, Kittikowit W, Sandhu I *et al.* Cell senescence in rat kidneys *in vivo* increases with growth and age despite lack of telomere shortening. *Kidney Int* 2003; **63**: 2134–2143.
22. Franceschi C, Bonafe M, Valensin S *et al.* Inflamm-aging. An evolutionary perspective on immuno-senescence. *Ann N Y Acad Sci* 2000; **908**: 244–254.
23. Baylis C, Fredericks M, Wilson C *et al.* Renal vasodilatory response to intravenous glycine in the aging rat kidney. *Am J Kidney Dis* 1990; **15**: 244–251.
24. Inamizu T, Chang MP, Makinodan T. Influence of age on the production and regulation of interleukin-1 in mice. *Immunology* 1985; **55**: 447–455.
25. Chen LC, Pace JL, Russell SW *et al.* Altered regulation of inducible nitric oxide synthase expression in macrophages from senescent mice. *Infect Immun* 1996; **64**: 4288–4298.
26. Spencer NF, Daynes RA. IL-12 directly stimulates expression of IL-10 by CD5+ B cells and IL-6 by both CD5+ and CD5- B cells: possible involvement in age-associated cytokine dysregulation. *Int Immunol* 1997; **9**: 745–754.
27. Castle S, Uyemura K, Wong W *et al.* Evidence of enhanced type 2 immune response and impaired upregulation of a type 1 response in frail elderly nursing home residents. *Mech Ageing Dev* 1997; **94**: 7–16.
28. Uyemura K, Castle SC, Makinodan T. The frail elderly: role of dendritic cells in the susceptibility of infection. *Mech Ageing Dev* 2002; **123**: 955–962.
29. White E, Hildemann WH, Mullen Y. Chronic kidney allograft reactions in rats. *Transplantation* 1969; **8**: 602–617.

30. Hancock WH, Whitley WD, Tullius SG *et al.* Cytokines, adhesion molecules, and the pathogenesis of chronic rejection of rat renal allografts. *Transplantation* 1993; **56**: 643–650.
31. Troppmann C, Gillingham KJ, Benedetti E *et al.* Delayed graft function, acute rejection, and outcome after cadaver renal transplantation. The multivariate analysis. *Transplantation* 1995; **59**: 962–968.
32. Troppmann C, Gillingham KJ, Gruessner RW *et al.* Delayed graft function in the absence of rejection has no long-term impact. A study of cadaver kidney recipients with good graft function at 1 year after transplantation. *Transplantation* 1996; **61**: 1331–1337.
33. Game DS, Lechler RI. Pathways of allorecognition: implications for transplantation tolerance. *Transplant Immunol* 2002; **10**: 101–108.
34. Lechler R, Ng WF, Steinman RM. Dendritic cells in transplantation-friend or foe? *Immunity* 2001; **14**: 357–368.
35. Sarwal M, Chua MS, Kambham N *et al.* Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. *N Engl J Med* 2003; **349**: 125–138.
36. Hippen BE, DeMattos A, Cook WJ *et al.* Association of CD20+ infiltrates with poorer clinical outcomes in acute cellular rejection of renal allografts. *Am J Transplant* 2005; **5**: 2248–2252.
37. Alausa M, Almagro U, Siddiqi N *et al.* Refractory acute kidney transplant rejection with CD20 graft infiltrates and successful therapy with rituximab. *Clin Transplant* 2005; **19**: 137–140.
38. Becker YT, Becker BN, Pirsch JD *et al.* Rituximab as treatment for refractory kidney transplant rejection. *Am J Transplant* 2004; **4**: 996–1001.
39. Steinmetz OM, Panzer U, Kneissler U *et al.* BCA-1/CXCL13 expression is associated with CXCR5-positive B-cell cluster formation in acute renal transplant rejection. *Kidney Int* 2005; **67**: 1616–1621.
40. Fuggle SV, Sanderson JB, Gray DW *et al.* Variation in expression of endothelial adhesion molecules in pretransplant and transplanted kidneys – correlation with intragraft events. *Transplantation* 1993; **55**: 17–23.
41. Ensminger SM, Spriewald BM, Steger U *et al.* Platelet-endothelial cell adhesion molecule-1 (CD31) expression on donor endothelial cells attenuates the development of transplant arteriosclerosis. *Transplantation* 2002; **74**: 1267–1273.
42. Denton MD, Davis SF, Baum MA *et al.* The role of the graft endothelium in transplant rejection: evidence that endothelial activation may serve as a clinical marker for the development of chronic rejection. *Pediatr Transplant* 2000; **4**: 252–260.
43. Turesson C. Endothelial expression of MHC class II molecules in autoimmune disease. *Curr Pharm Des* 2004; **10**: 129–143.
44. Tay SS, McCormack A, Rose ML. Effect of cognate human CD4+ T cell and endothelial cell interactions upon chemokine production. *Transplantation* 2004; **78**: 987–994.
45. Schmucker DL, Owen TM, Issekutz TB *et al.* Expression of lymphocyte homing receptors alpha4beta7 and MAdCAM-I in young and old rats. *Exp Gerontol* 2002; **37**: 1089–1095.
46. Masuda M, Takahashi H. Adhesion of leukocytes to endothelial cells in atherosclerosis. *Rinsho Byori* 1998; **46**: 1149–1155.
47. Cavallaro U, Castelli V, Del Monte U *et al.* Phenotypic alterations in senescent large-vessel and microvascular endothelial cells. *Mol Cell Biol Res Commun* 2000; **4**: 117–121.
48. Ogino T, Miura S, Komoto S *et al.* Senescence-associated decline of lymphocyte migration in gut-associated lymphoid tissues of rat small intestine. *Mech Ageing Dev* 2004; **125**: 191–199.
49. Diamond JR, Tilney NL, Frye J *et al.* Progressive albuminuria and glomerulosclerosis in a rat model of chronic renal allograft rejection. *Transplantation* 1992; **54**: 710–716.
50. Nickel P, Presber F, Bold G *et al.* Enzyme-linked immunosorbent spot assay for donor-reactive interferon-gamma-producing cells identifies T-cell presensitization and correlates with graft function at 6 and 12 months in renal-transplant recipients. *Transplantation* 2004; **78**: 1640–1646.